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Localization and Treatment of Transformed Tissues Using the Photodynamic Sensitizer 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a

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Background and Objective: **Photofrin is the photosensitizer currently used in most clinical trials examining the efficacy of photodynamic therapy (PDT) for the treatment and/or palliation of neoplasia. Although this drug has been shown to be efficacious in many of these trials, it possesses less than ideal qualities for use in a systemically administered photosensitizer. A new photosensitizer, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), was developed for PDT. HPPH possesses more rapid clearance from skin and greater cytotoxicity per drug dose than Photofrin. The aims of this study were to: (1) examine the uptake and retention of HPPH in tissues undergoing malignant transformation using laser-induced fluorescence, and (2) evaluate the efficacy of HPPH and 665 nm light in treating carcinogen-induced tumors of the hamster buccal cheek pouch.**

Study Design/Materials and Methods: **The model of tissue transformation was the carcinogen (9,10-dimethyl-1,2-benzanthracene)-induced premalignant and malignant lesions of the hamster buccal cheek pouch. Following induction of the specific transformation stages, hamsters were injected intraperitoneally with 0.5 mg/kg HPPH. Subsequently, the buccal mucosa was examined for fluorescence at various times up to 72 hours after photosensitizer injection.**

Results: **Uptake studies of HPPH showed highest fluorescence levels in tissues 48 hours after HPPH injection. Fluorescence levels of tissues increased significantly as follows. Normal < dysplasia < papillomas < squamous cell carcinomas. Carcinogen-induced tumors in 14 hamsters were treated with surface illuminations of red light (665 nm) via fiber optics coupled to an argon-ion pumped dye laser 48 hours after intraperitoneal injection with either 0.5 or 1.0 mg/kg HPPH. Complete necrosis of tumor tissues 7 days following PDT was observed in 57% (4/7) with 0.5 mg/kg and 86% (6/7) with 1.0 mg/kg HPPH.**

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Conclusions: It appears that HPPH may be a promising photosensitizer for the detection and photodynamic treatment of transformed tissues.

Key words: argon dye laser, diagnosis, hamster buccal cheek pouch, HPPH, laser-induced fluorescence, photodynamic therapy, Photofrin, photosensitization, squamous cell carcinoma

INTRODUCTION

Photodynamic therapy (PDT) is a form of cancer treatment that offers: (1) palliation for nonsurgical candidates with obstructive tumors, and (2) the possibility of curing noninvasive disease when treated in its earliest stages. PDT is a two-stage process with the first stage consisting of an injection of a photosensitizer. The photosensitizer most commonly used for this process is Photofrin (porfimer sodium; $\varepsilon \approx 2{,}500\ M^{-1}cm^{-1}$ at 630 nm, $\Phi_f \approx 0.03$ at 630 and 690 nm). The only side effect of Photofrin itself is cutaneous photosensitivity, which lasts ~4–6 weeks [1]. Following a period of time postinjection during which the photosensitizer is mostly cleared from a variety of tissues (24–72 hours) and retained in tumor, skin, and organs of the reticuloendotherial systems [2], the tumor is illuminated with 630 nm red light emanating from a laser, a process that constitutes the second and final stage of the therapy. The light can be coupled into quartz fiber optics having various specialized light delivery ends, which allow illumination of either superficial or interstitial disease. Tumor selectivity in treatment occurs through a combination of selective retention of photosensitizer, selective delivery of light, and drug/light dose selection such that normal tissues are relatively spared [3].

Many of the clinical reviews of PDT written in the past few years have concluded that this promising approach to treatment of solid malignant tumors requires better photosensitizers that may be more efficient than Photofrin and will not induce prolonged cutaneous photosensitivity [4,5]. As such, there is great interest in the synthesis of new sensitizing agents for use in PDT.

Pheophorbides and pyropheophorbides are photodynamic sensitizers that are effective against tumors grown in mice [6,7]. A specific pyropheophorbide, 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH), has shown antitumor efficacy in a murine tumor model and rapid clearance from skin [8]. This compound has a major absorption peak in the red part of the visible spectrum ($\varepsilon \approx 47{,}500\ M^{-1}cm^{-1}$ at 665 nm). As a result, greater photodynamic efficacy with a slight gain in penetration depth of light is obtained.

In parallel to the development of this new photosensitizer for clinical treatment purposes, HPPH also possesses a greater fluorescence emission peak at 671 nm ($\Phi_f \approx 0.3$), which can be exploited in studies examining drug localization and/or detection of malignant tissues using laser induced-fluorescence (LIF). Previous investigators have demonstrated the utility of LIF to monitor either drug levels and/or alterations in tissues [9–12].

The aims of this study were to: (1) examine the uptake and retention of HPPH in tissues undergoing malignant transformation using LIF, and (2) evaluate the efficacy of HPPH and 665 nm red light in treating carcinogen-induced tumors of the oral cavity. The animal model of tissue transformation was the dimethylbenzanthracene (DMBA)-induced premalignant and malignant lesions of the hamster buccal cheek pouch [13]. Of specific interest to this study were the developments of: (1) dysplastic leukoplakia at 6–8 weeks of continuous DMBA treatment, (2) carcinoma in situ (CIS) at 8–10 weeks of continuous DMBA treatment, (3) frank epidermoid carcinoma at 10–12 weeks of continuous DMBA treatment, and (4) large verrucous and invasive carcinoma at 12–14 weeks of continuous DMBA treatment [14].

MATERIALS AND METHODS

Photosensitizer

2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) was prepared by Dr. R. K. Pandey of Roswell Park Cancer Institute from methyl pyropheophorbide-a, which in turn was prepared from methyl pheophorbide-a isolated from *Spilulina sp.*, as described previously [6,7]. The compound was formulated in 5% dextrose water, 2% ethanol, and 0.1% polysorbate 80 for a final concentration of 1.1 mg/ml. The spectral characteristics of HPPH show an absorption band at 665 nm ($\varepsilon \approx 47{,}500\ M^{-1}cm^{-1}$) and peak emission at 671 nm ($\Phi_f \approx 0.3$, 20 nm full width half maximum bandwidth). The structure and spectral analysis of HPPH are shown in Figure 1.

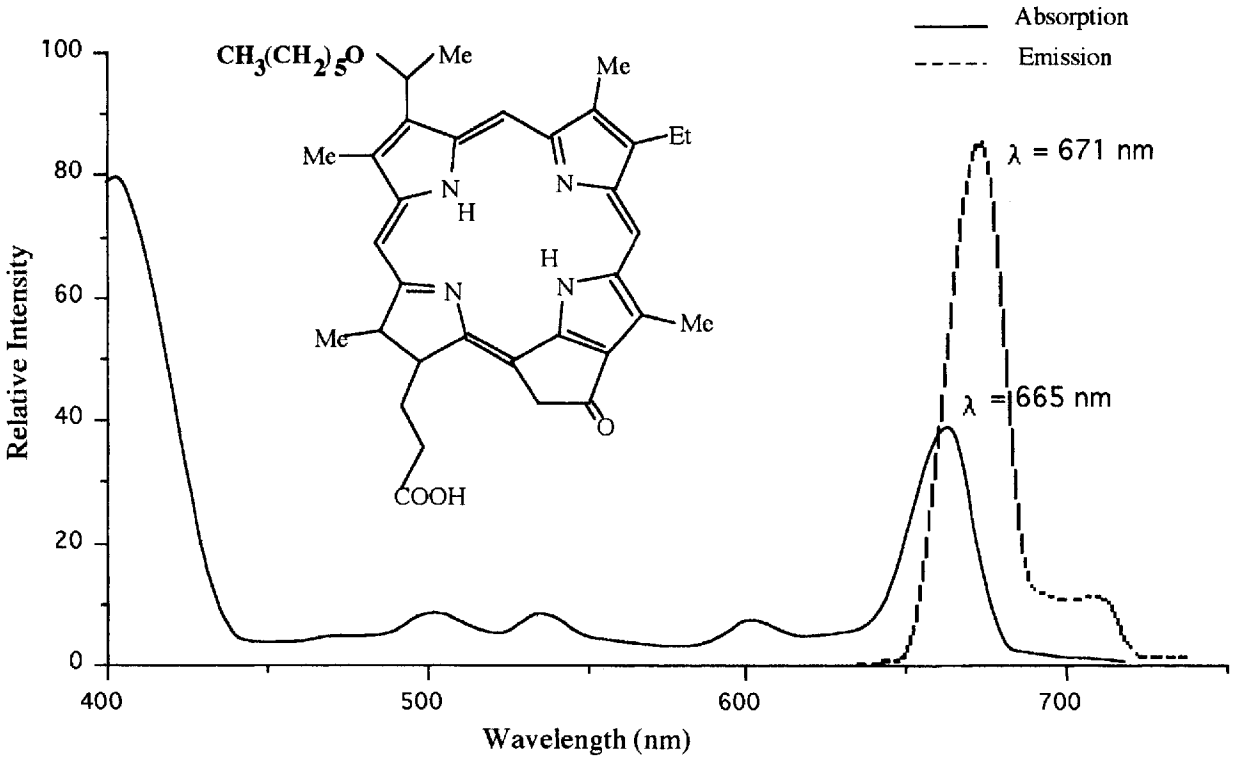

Fig. 1. Structure and spectral characteristics of hexylpyropheophorbide-a (HPPH). The spectral characteristics of HPPH show a longest absorption band at 665 nm and a peak emission at 671 nm.

Animal Model

Fluorescence localization and photodynamic studies were performed in the carcinogen (DMBA [9,10-dimethyl-1,2-benzanthracene])-induced hamster buccal cheek pouch tumor development model. This model shows consistent time patterns of tumor development as well as precancerous leukoplakic lesions. Male Syrian Golden hamsters (*Mesocricetus auratus*, retired breeders 150–200 g) were obtained from Charles River Laboratories (Kingston, Ontario, Canada). Hamsters were given chlorophyll-free food (Purina Mills, Richmond, IN) and tap water ad libitum.

For tumor induction, hamsters were lightly anesthetized using ethyl ether. With hamsters in a supine position, the buccal cheek pouches were everted. A 0.5% DMBA in acetone solution was applied with a camel hair brush to the exposed buccal mucosa and let set until the solution dried. Hamsters were treated three times per week with a day between DMBA treatments. Of specific interest to this study were the developments of: (1) dysplastic leukoplakia at 6–8 weeks of continuous DMBA treatment, (2) carcinoma in situ (CIS) at 8–10 weeks of continuous DMBA treatment, (3) frank epidermoid carcinoma at 10–12 weeks of continuous DMBA treatment, and (4) large verrucous and invasive carcinoma at 12–14 weeks of continuous DMBA treatment [14]. In addition to DMBA-treated tissues, untreated normal tissues (control) were also evaluated. Carcinogen-induced cancers were localized to the mucosal surface rather than invading deeply into the underlying connective tissues.

Experimental Protocol

For the fluorescence study, hamsters were divided into experimental and control (normal untreated buccal pouch mucosa) groups. The hamsters were injected intraperitoneally (i.p.) with 0.5 mg/kg HPPH at specific time points within the various stages of tumor development. Hamsters were sacrificed using an overdose of ethyl ether at 6, 24, 48, and 72 hours after injection. The buccal cheek pouches were then outstretched, exposed, and analyzed for specific fluorescence (n = 9 at each time point). In a separate group of hamsters without HPPH (i.e., 0 hour; uninjected, n = 9), fluorescence of the diseased oral mucosa was evaluated to examine the pres-

ence of endogenous fluorophores. The fluorescence detection instrumentation has been described previously [9]. In brief, HPPH fluorescence in tissue was detected by a nonimaging silicon photodetector at 690 nm (10 nm bandwidth) after simultaneous excitation at 612 and 632 nm. Mucosal tissues adjacent to and within the field exposed to DMBA (which included areas of neoplastic tissue), normal appearing oral mucosa, and areas visually suspicious for disease (including hyperfluorescent foci) were biopsied after fluorescence measurements. Biopsied samples were used for histopathological examination.

For the photodynamic treatment study, two groups of hamsters (n = 7 each group) that received applications of DMBA to the oral mucosa and subsequently developed tumors (invasive SCC or papillomas with severe dysplasia) were injected i.p. with either 0.5 or 1.0 mg/kg of HPPH. Forty-eight (48) hours after photosensitizer injection, hamster buccal pouches were everted, outstretched, and tumors to be treated with light selected from the DMBA-treated field (which may have had several tumors within the same field). For controls, three groups of hamsters (n = 2 each group) that did not previously receive application of DMBA (i.e., complete normal mucosa) were exposed to light application at the same time points following injection of either 0.5 or 1.0 mg/kg of HPPH, or without injection of HPPH. Anesthesia was performed during the PDT treatment using sodium pentobarbital (50 mg/kg i.p. injection). Eligibility requirements of tissues destined for photodynamic treatment were as follows: (1) tumorous tissue consisting of either invasive squamous cell carcinoma or papillary tumors with severe dysplasia, (2) tumor volume $< 400\ mm^3$ (size < 10 mm), and (3) no evidence of necrotic or hemorragic tissues. Exposure of the oral tissues for light treatment was performed using tongue depressors and forceps. Light irradiation of the surface was then performed over an area encompassing the tumor with a treatment margin of 2–3 mm. Surface illumination was performed using 400 μm core quartz fiber optics with a graded index lens providing isotropic distribution of the light at one end. The other ends of the fiber optics were coupled to a dye laser (Spectra Physics, Mountainview, CA), which was pumped by an argon ion laser (Spectra Physics). The dye laser that contained kiton red solution was tuned for an output wavelength of 665 nm. At both drug doses tested, the dose rate of light illumination was 100 mW/cm^2 for a total light dose of 150 J/cm^2.

PDT-treated tissues were followed for 7 days after light illumination. Tumor volumes were measured after treatment and compared to pretreatment values. The animals were sacrificed at day 7, treated lesions excised and sent for histological examination to determine PDT efficacy. In three separate animals treated with PDT (light and 1.0 mg/kg HPPH), the buccal mucosal tissues were followed out to 4 weeks after light treatment to observe tissue healing and/or tumor recurrence.

Seven (7) day tumor response was classified as follows: (1) initial complete response (I-CR), 100% reduction of tumor volume, (2) initial partial response (I-PR), >50% reduction of tumor volume, (3) no significant change (NC), <50% reduction of tumor volume, (4) progression of disease (PD), >25% increase of tumor volume.

Histopathological Examination

The excised tissues, which were previously either examined fluorometrically or treated, were placed in 10% formalin for routine histological preparation and hematoxylin and eosin staining. Histopathological examination for the presence of invasive squamous carcinoma, papillary disease, dysplasia, or necrotic tissue was performed on each sample by a pathologist blinded as to the fluorescence results and treatment parameters.

Statistical Analysis

All data are presented as mean ± standard deviation. Statistical significance between mean fluorescence values and/or slopes was analyzed by one-way analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons Tests using GraphPad Instat (San Diego, CA). Determinations of slopes were performed by Linear Regression and Spearman Rank Correlation Tests using GraphPad Instat.

RESULTS

Fluorescence Measurements of DMBA-Induced Lesions as a Function of Time After HPPH Administration

A primary objective of this study was to examine the uptake and retention of HPPH in tissues exposed to a carcinogenic process after i.p. injection of HPPH (0.5 mg/kg) to the animals. The oral mucosal tissues that had developed premalignant and malignant diseases, based upon the

TABLE 1. Fluorescence Levels of HPPH in Transformed Tissues*

Time	Tissue transformation				Time	*P* value		
	Normal	Dysplasia	Papilloma	SCC		S vs. P	S vs. D	P vs. D
0 h	0.01 ± 0.01 (4)	0.03 ± 0.03 (5)	0.09 ± 0.05 (12)	0.33 ± 0.06 (5)	0 h	<0.001	<0.001	ns
6 h	—	0.09 ± 0.03 (5)	0.40 ± 0.09 (6)	0.63 ± 0.17 (8)	6 h	<0.01	<0.001	<0.01
24 h	—	0.18 ± 0.08 (7)	0.47 ± 0.17 (9)	0.81 ± 0.15 (3)	24 h	<0.01	<0.001	<0.01
48 h	0.09 ± 0.05 (4)	0.35 ± 0.17 (11)	0.58 ± 0.33 (15)	1.04 ± 0.32 (11)	48 h	<0.001	<0.001	ns
72 h	—	0.27 ± 0.08 (8)	0.45 ± 0.18 (18)	0.64 ± 0.18 (6)	72 h	<0.05	<0.001	<0.05

*Fluorescence measurements were performed at each time point after 0.5 mg/kg i.p. injection. Data represent the mean (volts) ± SD, () = number of samples. The probabilities were calculated by Tukey-Kramer Multiple Comparisons Tests. S, squamous cell carcinoma; P, papilloma; D, dysplasia; ns, not significant.

number of applications of DMBA as well as confirmed by histopathological examination, were examined at various times for fluorescence using the nonimaging photodetector. Fluorescence levels of HPPH in transformed tissues are shown in Table 1. Significantly greater fluorescence was seen in normal mucosa of animals (n = 4) 48 hours postinjection with HPPH vs. normal mucosa of animals (n = 4) not injected with the photosensitizer (injected, 0.09 ± 0.05 volts vs. uninjected, 0.01 ± 0.01 volts, $P < 0.001$). This level of significance suggests that small quantities of HPPH are taken up and retained in normal buccal mucosa at 48 hours after drug administration.

Figure 2A shows the fluorescence levels in DMBA-induced dysplastic lesions (n = 36) detected in vivo by the dual wavelength excitation photodetector as a function of time post-HPPH administration. Fluorescence levels of DMBA-induced dysplastic lesions as a function of time after HPPH injection were significantly different from one another as determined by ANOVA ($P < 0.0001$). The rate of fluorescence increase at 48 hours after drug injection was 0.007 ± 0.001 volts/hour (correlation coefficient r = 0.72). Dysplastic lesions in uninjected animals (time 0) demonstrated endogenous fluorescence levels of 0.03 ± 0.03 volts, which were less than fluorescence levels of dysplastic lesions in animals at 6 and 24 hours after HPPH injection (6 hours, 0.09 ± 0.03 volts; 24 hours, 0.18 ± 0.08 volts; $P > 0.05$), but the differences were not significant. However, significant differences in fluorescence levels of dysplastic lesions in HPPH-injected versus uninjected animals were seen 48 and 72 hours after drug administration [uninjected, 0.03 ± 0.03 volts vs. 0.35 ± 0.17 volts (48 hours), $P < 0.001$; 0.27 ± 0.08 volts (72 hours), $P < 0.01$]. The maximum fluorescence levels of tissues with mild-to-moderate dysplasia were observed 48 hours after drug injection, which were significantly greater than fluorescence levels of dysplastic lesions at 6 ($P < 0.01$) or 24 ($P < 0.05$) hours after HPPH administration and normal tissues 48 hours after photosensitizer injection ($P < 0.01$).

Figure 2B shows the fluorescence levels of DMBA-induced papillary tumors with severe dysplasia (n = 60) detected in vivo vs. various times after HPPH administration. Fluorescence levels of DMBA-induced papillary lesions at various times after HPPH injection were significantly different from one another as determined by ANOVA ($P < 0.0001$). The rate of fluorescence increase up to 48 hours after drug injection was 0.009 ± 0.002 volts/hour (correlation coefficient r = 0.61). Papillary tumors in uninjected animals (time 0) demonstrated endogenous fluorescence levels of 0.09 ± 0.05 volts, which were significantly less than fluorescence levels of papillary tumors in animals at 6, 24, 48, and 72 hours after HPPH injection (6 hours, 0.40 ± 0.09 volts, $P < 0.05$; 24 hours, 0.47 ± 0.17 volts; $P < 0.01$; 48 hours, 0.58 ± 0.33 volts, $P < 0.001$; 72 hours, 0.45 ± 0.18 volts, $P < 0.001$). Maximum fluorescence levels of papillary tumors with severe levels of dysplasia were observed 48 hours after drug injection, similar to mild-to-moderate dysplastic lesions.

Figure 2C shows the fluorescence levels of DMBA-induced squamous cell carcinomas (n = 33) detected in vivo vs. various times after HPPH administration. Fluorescence levels of DMBA-induced invasive cancers at various times after HPPH injection were significantly different from one another as determined by ANOVA ($P < 0.0001$). The rate of specific fluorescence increase up to 48 hours after injection was 0.011 ± 0.002 volts/hour (correlation coefficient r = 0.74). Squamous cell carcinomas in uninjected animals (time 0) demonstrated endogenous fluorescence levels of 0.33 ± 0.06 volts, which were not significantly less than fluorescence levels of squamous cell car-

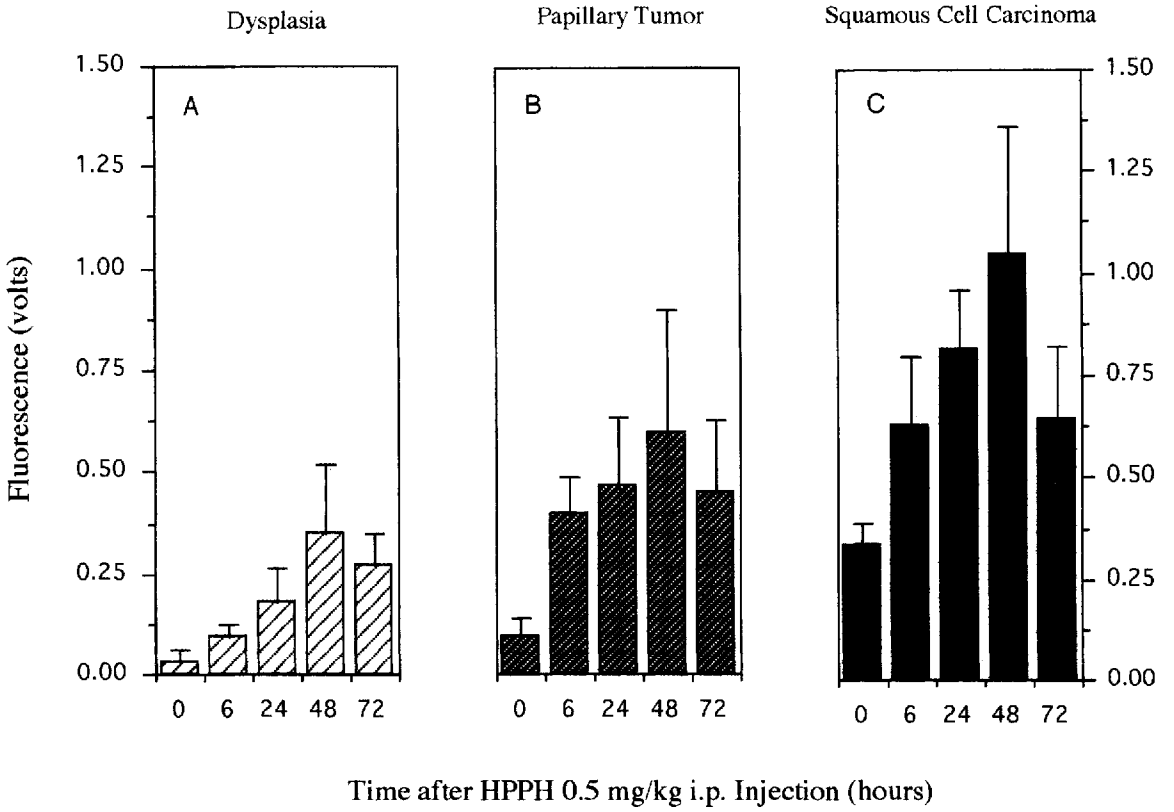

Fig. 2. Fluorescence (mean volts ± standard diviation) of DMBA-induced lesions detected in vivo by the fluorescence photometer vs. various times after 0.5 mg/kg HPPH (hours): **A**, dysplasia; **B**, papillary tumor, **C**, squamous cell carcinoma.

cinomas in animals at 6, 24, and 72 hours after HPPH injection (6 hours, 0.63 ± 0.17 volts; 24 hours, 0.81 ± 0.15 volts; 72 hours, 0.64 ± 0.18 volts; all at $P > 0.05$). However, significantly greater fluorescence levels of invasive cancers in HPPH-injected animals 48 hours after drug administration were seen vs. uninjected controls (uninjected, 0.33 ± 0.06 volts vs. 1.04 ± 0.32 volts, $P < 0.001$). The time of maximum accumulation of HPPH in invasive cancers, as measured by fluorescence, was 48 hours after injection. This time point was significantly greater than fluorescence levels of invasive cancers in other animals at 6 ($P < 0.01$) or 72 ($P < 0.05$) hours after HPPH administration, but not significantly greater than fluorescence levels of cancers at 24 hours after drug injection ($P > 0.05$).

Fluorescence Measurement of DMBA-Induced Lesions as a Function of Histopathology

Fluorescence levels of carcinogen-induced lesions were compared to one another to examine the relationship between histopathology and fluorescence over the time course examined (see Fig 2: A vs. B vs. C). All lesions examined (i.e., dysplasia, papillary tumors, and invasive cancers) demonstrated significant ($P < 0.0001$) increases in fluorescence levels in tissues up to 48 hours after HPPH injection (dysplasia, 0.007 ± 0.001 volts/hour; papillary disease, 0.009 ± 0.002 volts/hour; squamous cell carcinoma, 0.011 ± 0.002 volts/hour). However, the slopes were considered by ANOVA to be not significantly different from one another ($P = 0.11$). At all time points examined after HPPH injection, squamous cell carcinomas demonstrated significantly greater fluorescence levels than papillary tumors and dysplastic lesions, and fluorescence levels of papillary tumors with severe dysplasia were significantly greater than dysplastic lesional fluorescence levels at all time points post-HPPH injection, except 48 hours, which were not significantly different ($P > 0.05$) as shown in Table 1. Thus total HPPH accumulation, as determined by fluorescence, is highly dependent upon the transformation stage of the tissues.

Detection of Endogenous Chromophores in DMBA-Induced Lesions

Endogenous fluorescence detected in DMBA-induced lesions was considered by ANOVA to be significant at the $P < 0.0001$ level. Background fluorescence levels of squamous cell carcinomas in uninjected animals were significantly greater than fluorescence levels of papillary tumors and dysplastic lesions in uninjected control animals (squamous cell carcinoma, 0.33 ± 0.06 volts vs. papillary, 0.09 ± 0.05 volts, $P < 0.001$; and dysplasia, 0.03 ± 0.03 volts, $P < 0.001$). This suggests the presence of endogenous chromophores in the invasive cancer tissues of the hamster model that are emitting fluorescence at ~690 nm upon excitation at 632 nm.

Photodynamic Treatment of Carcinogen-Induced Tumors

Fourteen (14) hamsters that had their buccal mucosa exposed to DMBA continuously (3 times per week) for a period of 10–14 weeks to produce invasive SCC and/or papillary tumors with severe dysplasia were randomly divided into two groups. One group of animals received an intraperitoneal (i.p.) injection of 0.5 mg/kg HPPH; the second group received a 1.0 mg/kg i.p. injection. Tumors, which were selected from areas of multifocal involvement prior to irradiation, were then illuminated with 665 nm red light 48 hours after photosensitizer injection. The range of tumor volume was 12–360 mm^3 in the 0.5 mg/kg injection group, 100–343 mm^3 in the 1.0 mg/kg injection group. The oral mucosal tumors in both groups of animal received the same total light dose of 150 J/cm^2 (power density: 100 mW/cm^2) via surface illumination.

The results of tumor response to PDT with 0.5 mg/kg or 1.0 mg/kg HPPH are shown in Table 2. Tumor response to PDT 7 days after light application in seven tumors treated with PDT in the 0.5 mg/kg injection group demonstrated an initial complete response with evidence of total tumor necrosis occurring in 4 (57%) of seven cheek pouch tumors, whereas two tumors showed no observable change in tumor volume and 1 tumor showed progression of disease 7 days post-PDT. Tumor response in the group exposed to light 48 hours after 1.0 mg/kg HPPH demonstrated an improvement in tumor eradication over the lower drug dose. Complete necrosis of tumor in response to PDT in animals that had received an injection of 1.0 mg/kg HPPH prior to surface illumination was seen in 6 (86%) of 7 cheek pouch tumors, whereas one tumor showed no observable change in tumor volume 7 days post-PDT.

All cheek pouches that demonstrated eventually complete necrosis in both groups became edematous within 24 hours after light application. Thereafter, degeneration and a decrease in tumor volumes were observed selectively after several days. Mucosal edema was not observed in cheek pouches that did not respond to PDT. An obvious relationship between tumor response and tumor volume was not observed. In the control group (hamsters without injection of HPPH), no changes in normal mucosa following exposure to light were observed. Other control group of hamsters with 0.5 mg/kg HPPH injection showed slight edema in normal mucosa within 24 hours after light application, which disappeared by 48 hours posttreatment. Group of hamsters given 1.0 mg/kg HPPH injection demonstrated moderate edema in normal mucosa within 24 hours after light application. The moderate edema in normal mucosa decreased gradually within several days. The mucosa with moderate edema, however, demonstrated some slight transient focal changes at 7 days after light application.

Histopathological examination of the treated area (100 mW/cm^2, 150 J/cm^2) 2 days post-PDT showed extensive necrosis in which the tissues demonstrated hemorrhage, infiltration of leukocytes, and degenerated tumor cells (Fig. 3). The untreated tumorous area demonstrated invasive SCC as determined by histopathological examination (Fig. 4). A follow-up was performed over the course of 1 month post-PDT on the cheek pouch tissues of three animals in which total necrosis of the treated area was observed. The process of tumor eradication and wound healing in the treated cheek pouches was as follows: (1) tissues became severely edematous within 24 hours after light treatment, (2) degeneration and necrosis of tumor tissues were observed selectively 2–3 days after PDT, (3) total necrosis of treated tissues 7 days after PDT, (4) ulceration and contraction of mucosal tissues from weeks 1–2 after PDT, and (5) wound healing and scar formation 4 weeks after therapy. Tumor recurrences were not observed beyond 4 weeks after PDT.

DISCUSSION

The present study examined the fluorescence and photosensitizing abilities of a new photodynamic sensitizer, HPPH, in oral tissues of

TABLE 2. Results of PDT for Hamster Buccal Cheek Pouch Tumors*

Animal #	Injection (mg/kg)	Tumor volume		Edema	Tumor response on days 7
		Pre-PDT (mm^3)	Post-PDT (mm^3)		
1	0.5	12	0	+ +	I-CR
2	0.5	27	80	−	PD
3	0.5	45	0	+	I-CR
4	0.5	96	0	+ +	I-CR
5	0.5	150	150	−	NC
6	0.5	336	0	+	I-CR
7	0.5	360	420	−	NC
8	1.0	100	0	+ +	I-CR
9	1.0	105	0	+ +	I-CR
10	1.0	120	0	+ +	I-CR
11	1.0	168	0	+ +	I-CR
12	1.0	300	0	+	I-CR
13	1.0	315	378	−	NC
14	1.0	343	0	+ +	I-CR
15	0.0	0	—	−	—
16	0.0	0	—	−	—
17	0.5	0	—	±	—
18	0.5	0	—	±	—
19	1.0	0	—	+	—
20	1.0	0	—	+	—

*Seven (7) days tumor response to HPPH (either 0.5 or 1.0 mg/kg) 48 hours after i.p. injection and 665 nm light (total energy: 150 J/cm^2, power density: 100 mW/cm^2). Control groups: #15-#20 (normal mucosa). Symbols indicate the presence or absence of edema in cheek pouch at 24 hours after PDT treatment. (−): no edema, (±): slight edema, (+): moderate edema, (+ +): sever edema. Tumor response was evaluated according to the following criteria: (1) I-CR, initial complete response; (2) I-PR, initial partial response; (3) NC, no significant change; and (4) PD, progression of disease.

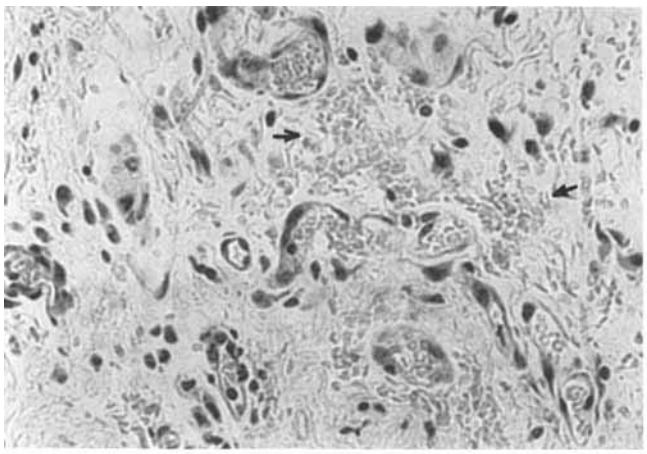

Fig. 3. Hematoxylin and eosin stained section (×400) of the treated area (power density: 100 mW/cm^2, total energy: 150 J/cm^2) 2 days post-PDT showing extensive necrosis in which the tissue was hemorragic (arrow) and infiltrated leukocytes, but devoid of viable tumor cells.

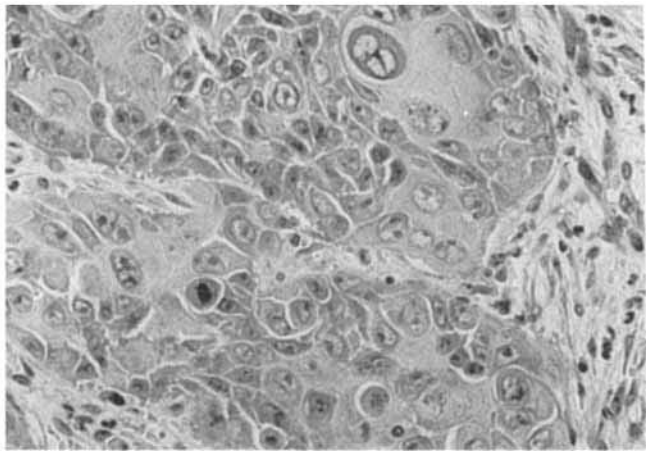

Fig. 4. Hematoxylin and eosin stained section (×400) of the untreated tumorous area demonstrating invasive SCC. Cells have large nuclear-cytoplasmic ratios and some are binuclear.

hamsters undergoing malignant transformation. Previous uptake studies in mice by Bellnier et al. [8] using [^{14}C] HPPH demonstrated that a photosensitizer ratio of 4.5 in tumor vs. normal contralateral skin was obtained 24 hours after drug injection. Compared to the normal tissue response of 630 nm light and Photofrin, the currently used photosensitizer in PDT, normal skin photosensitivity to HPPH and 665 nm was significantly reduced [8]. Tumor response at 7 days and 30 days to 665 nm light (150 J/cm^2) 24 hours after injection of 0.3 mg/kg HPPH was reported to be 100% and 58%, respectively [8]. Based upon these ob-

servations as well as others [6,7], HPPH shows promise as a photosensitizer for clinical development.

In this study, we tested our ability to detect the photosensitizer in tissues using LIF at several times after injection of 0.5 mg/kg HPPH. The model of tissue transformation used was the hamster buccal cheek pouch undergoing premalignant and malignant transformation. Application of 0.5% DMBA in acetone produces multifocal areas of disease involvement depending upon the length of carcinogen application. Previous fluorescence studies indicated that Photofrin was taken up and retained in tissues of the cheek pouch with a dependency on the transformation stage [9]. Our a priori assumption was that HPPH would demonstrate results similar to Photofrin in the same animal model. All carcinogen-induced lesions that were examined (i.e., dysplastic lesions, papillomas with significant levels of dysplasia, and SCC) demonstrated fluorescence levels that were maximum 48 hours after photosensitizer injection. Uptake studies using [^{14}C] demonstrated previously that subcutaneously implanted radiation induced fibrosarcomas (RIF1) in murine model showed maximal concentrations of photosensitizer seven hours after injection [8]. However, it is difficult to compare these kinetic data with those data obtained by fluorescence measurement in this study because animal species, characteristics of tumor (i.e., implanted or spontaneous), tumor histology and injection route were entirely different. In addition, invasive SCC demonstrated significantly greater fluorescence levels than papillary tumors and dysplasia at all time points examined. Likewise, papillary tumors were significantly greater in fluorescence than dysplastic lesions at most time points as well. Thus, HPPH accumulation, as determined by fluorescence, is dependent upon the transformation stage of the tissues in the increasing order of normal mucosa, mild to moderate dysplasia, papilloma with severe dysplasia, and squamous cell carcinoma. These results are similar to those previously demonstrated in the same animal model using Photofrin [9].

Other reasons for the increase fluorescence levels are unknown. Further studies in this cancer model will be needed to examine determinants of tissue fluorescence. These include examination of several parameters determining fluorescence signals such as (1) tissue absorption and scattering properties at the excitation and emission wavelength, (2) tissue and spatial distribution of HPPH within the detection volumes, and (3) background contribution to the emitted signals by endogenous chromophores.

Endogenous fluorescence detected in the DMBA-induced lesions was considered to be significant. This suggests the presence of endogenous chromophores in the invasive cancer tissues of the hamster model that are emitting fluorescence at 690 nm. Harris and Werkhaven [15] noted the presence of endogenous porphyrins restricted to the surface of ulcerated tumors which emitted 690 nm fluorescence after ultraviolet excitation in the hamster buccal pouch model [15]. This autofluorescence was attributed to the presence of the several different species of bacteria inhabiting ulcerated and necrotic tissues. Yuanlong et al. [16] detected endogenous porphyrins in buccal carcinomas by using either a photomultiplier tube or optical multichannel analyzer, which detected fluorescence emission at 630 and 690 nm after 365 nm excitation. In addition, Weagle et al. [17] noted the presence of a chlorophyll compound derived from rodent food that emitted a prominent 674 nm peak. In this study, we attempted to eliminate endogenous fluorescence by: (1) using chlorophyll-free animal food, (2) disregarding necrotic tissues tissues inhabited by bacteria on their surfaces, and (3) using background subtraction.

The observations that HPPH was taken up and retained to a greater extent in malignant and premalignant tissues than in normal tissues provided an impetus for examining the efficacy of PDT using this second-generation photosensitizer in the treatment of tumor tissues. Complete tumor necrosis of most buccal cheek pouch tumors was evident 7 days after PDT (150 J/cm^2, 665 nm). Of the treated lesions in hamsters injected with 0.5 mg/kg HPPH, 57% displayed PDT-induced necrosis. The percentage of treated lesions resulting in complete necrosis increased to 86% in animals injected with 1.0 mg/kg. We estimated PDT efficacy for tumors with different volumes using 0.5 or 1.0 mg/kg HPPH. Relatively large tumors (360, 315, and 150 mm^3) and one small tumor (27 mm^3) showed treatment failures in both groups. There was no obvious relationship between PDT efficacy and tumor volume (Table 2). All cheek pouches that demonstrated complete necrosis in both groups showed moderate or severe edema within 24 hours after light application. Thereafter, degeneration and decrease in tumor volumes were observed selectively after several days. In the control group with 0.5 mg/kg HPPH injection and

light application, normal mucosa showed slight edema. Mucosal edema was not observed in cheek pouches in the treatment groups that did not respond to PDT.

A total of four tumors in both drug dose groups resulted in treatment failure. It is believed that the drug accumulation in these tissues was below the threshold drug dose since they did not demonstrate any mucosal edema, which is induced by photodynamic reaction in responding tissues. The cause of treatment failures may be explained simply by failure of intraperitoneal injection that may occur infrequently. In this study, PDT efficacy is accompanied by the presence of mucosal edema after laser illumination. Tumors that reacted to the drug and light did so in a manner similar to that observed by others using Photofrin and 630 nm light in this animal model [18]. Treated tissues became edematous, followed by degenerative and necrotic changes. Hemorrhage and infiltration of leukocytes were also observed. The combination of HPPH and light has vascular cytotoxicity on treated tumors, similar to vascular shutdown associated with Photofrin in murine models [19]. Further work will be required in this animal tumor model to determine: (1) the therapeutic light and drug doses for long-term control of treated lesions, and (2) sites and/or mechanism of damage. A therapeutic role for HPPH and light in treating cancers of the oral cavity is suggested.

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